A NOVEL HANSENULA POLYMORPHA GENE CODING FOR ALPHA 1,6-MANNOSYLTRANSFERASE AND PROCESS FOR THE PRODUCTION OF RECOMBINANT GLYCOPROTEINS WITH HANSENULA POLYMORPHA MUTANT STRAIN DEFICIENT IN THE SAME GENE

Technical Field

The present invention relates to a novel *Hansenula polymorpha* gene coding for α-1,6-mannosyltransferase initiating outer chain elongation, an *H. polymorpha* mutant strain having a deficiency in the gene, and a process for producing a recombinant glycoprotein using such a mutant strain.

Background Art

Upon large-scale expression of therapeutic proteins, according to characteristics of host cells or target proteins, a target protein may vary in expression levels, water solubility, expression sites, modification, and the like. Thus, the most suitable expression system for a target protein must be selected to establish an effective production system.

Most therapeutic proteins are glycoprotiens where oligosaccharides are covalently bonded to asparagine residues as they pass through the endoplasmic reticulum (ER) and Golgi apparatus (Jenkins et al., Nat. Biotechnol., 14, 975-9, 1996). The structure and kind of sugar moieties

greatly affect folding, biological activity and stability in serum of glycoprotiens. Thus, to date, for producing therapeutic recombinant glycoproteins having authentic sugar moieties and therapeutic activity, the most commonly used approach is to use animal cell expression systems. However, there are drawbacks to animal cell culture systems, which include low yield, high cost due to expensive culture media, retroviral contamination, and a long period of time required for establishing stable cell lines. Thus, animal cell culture systems have limited applications in producing recombinant glycoproteins. In this regard, many attempts have been made to use, as an alternative to animal cell expression systems, yeast expression systems, which are unicellular eukaryotes and share the early steps of the N-linked glycosylation pathway of higher animal cells, to produce recombinant glycoproteins of medical importance.

Microbial eukaryotes such as yeasts have advantages of rapidly producing high-yield proteins, utilizing sterilized and well-controlled production conditions, being easily genetically engineered, having no risk of infections by human or animal pathogens, and ensuring easy protein recovery. However, oligosaccharides attached to proteins synthesized in yeasts have different type of sugar moieties from those of target organisms such as mammalians, and thus may cause immune responses in animal cells. Also, this yeast-specific outer chain glycosylation of the high mannose type, also denoted hyperglycosylation, brings rise to heterogeneity of a recombinant protein product, which

may make the protein purification complicated or difficult. Further, the specific activity of enzymes may be lowered due to the increased carbohydrate level (Bekkers et al., Biochem. Biophy. Acta. 1089, 345-351, 1991).

To solve the above problems, there is a need for glycotechnology which introduces into yeasts a glycosylation pathway of animal cells capable of producing glycoproteins having identical biological activity to those derived from mammalians.

When recombinant glycoproteins are expressed in traditional yeast, *Saccharomyces cerevisiae*, the addition of a series of 50 to 200 mannose residues to a core oligosaccharide, resulting in hypermannosylation, and the presence of α-1,3-linked terminal mannose recognizable as an antigen in the body were viewed as large constraints in employing the yeast as a host for glycoprotein production (Dean, Biochim. Biophys. Acta., 1426, 309-322, 1999; Ballou, Methods Enzymol., 185, 440-444, (1990)). By contrast, when recombinant glycoproteins are expressed in the methylotropic yeasts, *Hansenula polymorpha* and *Pichia pastoris*, they are expressed in a hypermannosylated form compared to natural forms, but the overall length of mannose outer chains is shorter than those expressed in *S. cerevisiae* (Kang et al., Yeast 14, 371-381, 1998; Kim et al., Glycobioloby, 14, 243-251, 2004; Bretthauer and Castellino, Biotechnol. Appl. Biochem. 30, 193-200, 1999). In particular, since sugar chains synthesized in the methylotrophic yeasts, *H. polymorpha* and *P. pastoris*, do not contain the strongly

immunogenic α-1,3-linked terminal mannose (Kim et al., Glycobioloby, 14, 243-251, 2004; Montesino et al., Protein Expr. Purif. 14, 197-207, 1998), the methylotrophic yeasts are considered superior host systems to traditional yeast, *S. cerevisiae*, for the production of glycoproteins having therapeutic value in humans.

Many attempts were made in the glycotechnology field to develop hosts capable of producing therapeutic recombinant glycoproteins containing human compatible sugar chains using *P. pastoris* and *S. cerevisiae* (Chiba et al., J. Biol. Chem., 273, 26298-26304, 1998; Callewaert et al., FEBS Lett., 503, 173-178, 2001; Choi et al., Proc. Natl. Acad. Sci. U S A, 100, 5022-5027, 2003; Hamilton et al., Science, 301, 1244-1246, 2003). For example, an attempt was made to produce a glycoprotein where an intermediate including the human mannose-type $Man_5GlcNAc_2$ N-glycan was attached using a recombinant *S. cerevisiae* obtained by further genetically manipulating a triple mutant yeast (*och1Δmnn1Δmnn4Δ*) to express mammalian α-1,2-mannosidase in the ER (Chiba et al., J. Biol. Chem., 273, 26298-26304, 1998). The triple mutant has disruption in three genes: *OCH1* that plays a critical role in outer chain initiation (Nakanishi-Shindo et al., J. Biol. Chem. 268, 26338-26345, 1993; US Patent 5,705,616; US Patent 5,798,226); *MNN1* that mediates addition of the immunogenic α-1,3-linked terminal mannose (Gopal and Ballou, Proc. Natl. Acad. Sci. USA 84, 8824, (1987); US Patent 5,135,854); and *MNN4* that addes phosphates to a sugar chain (Jigami and Odani, Biochim. Biophys. Acta., 1426, 335-345,

1999). In addition, according to recent studies (Choi et al., Proc. Natl. Acad. Sci. U S A, 100, 5022-5027, 2003; Hamilton et al., Science, 301, 1244-1246, 2003), host developments in *P. pastoris* were made to produce recombinant glycoproteins with the human complex-type *N*-glycan $GlcNAc_2Man_3GlcNAc_2$ by introducing five different enzymes derived from eukaryotes into a secretory pathway in order to introduce the human glycosylation pathway into mutant strains (Japanese Pat. 07145005; Japanese Pat. 07150780; International Pat. Publication WO 0200856 A2; International Pat. Publication WO 0200879 A2) which have a disruption in the *OCH1* gene mediating outer chain initiation. However, to date, from the viewpoint of glycotechnolgy, attempts have rarely been made to produce recombinant glycoproteins with human-type sugar chains in the methylotropic yeast *H. polymorpha* which is gaining popularity as a host for the expression of therapeutic recombinant proteins since it has been employed for producing hepatitis vaccines.

As described in Korean Pat. Application No. 2002-37717, the present inventors, before the present invention, cloned the *OCH1* gene playing a critical role in the outer chain synthesis of *H. polymorpha*, establishing a mutant strain (*Hpoch1*Δ) having a disrupted *OCH1* gene, and developed a process for producing a recombinant glycoprotein with a sugar chain structure closer to a natural form by preventing hyperglycosylation using such a mutant. However, in the *Hpoch1*Δ mutant strain having a disruption in the *OCH1* gene of *H. polymorpha*, outer chain glycosylation is still

initiated by α-1,6-mannose linkage. Thus, there is a need for the finding of a gene coding for α-1,6-mannosyltransferase and prevention of the above human incompatible glycosylation pathway.

Disclosure of the Invention

With an aim to overcome the above problems and develop a production system for a recombinant glycoprotein having therapeutic value in humans using a methylotrophic, thermotolerant yeast *Hansenula polymorpha* that is widely used as a host system for mass expression of various heterogeneous genes, the present inventors cloned a novel gene *HpOCH2* in a *H. polymorpha* strain DL-1 using information based on the complete genome information for *H. polymorpha*, identified that the novel gene has the activity of α-1,6-mannosyltransferase responsible for the outer chain initiation, and developed a mutant strain having a disruption in the above gene. Then, the present inventors found that the mutant strain prevents a human incompatible glycosylation pathway, and, when a heterogeneous sugar chain-modifying enzyme is expressed in the mutant strain, is capable of producing a recombinant glycoprotein with a human mannose-type N-glycan $Man_5GlcNAc_2$ other than a yeast-specific *N*-glycan, thereby leading to the present invention.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows a nucleotide sequence of *H. polymorpha HpOCH2* gene and its predicted amino acid sequence, wherein a transmembrane spanning region is underlined, and an amino acid sequence corresponding to a DXD element is bold and underlined;

Fig. 2 is a multiple alignment of amino acid sequences of Och1 protein homologues of *H. polymorpha* and other yeast strains (HpOch2p: *H. polymorpha* Och2 protein; HpOch1p: *H. polymorpha* Och1 protein; CaOch1p: *Candida albicans* Och1 protein; PpOch1p: *Pichia pastoris* Och1 protein; ScOch1p: *Saccharomyces cerevisiae* Och1 protein; and SpOch1p: *Schizosaccharomyces pombe* Och1 protein), wherein parenthesized numerals indicate amino acid identity between *H. polymorpha* HpOch2p and Och1p analogues of other yeast strains;

Fig. 3 is a diagram for inducing disruption of the *H. polymorpha HpOCH2* gene by *in vivo* DNA recombination;

Fig. 4 shows growth properties of an *H. polymorpha Hpoch2*△ mutant strain, wherein cultures (OD_{600}=1) of a *H. polymorpha* wild type and two mutant strains, *Hpoch1*△ and *Hpoch2*△, which had arrived at an exponential phase, were 10-fold serially diluted, and 3 μl of each dilution was spotted on a YPD medium and cultured for two days (A: YPD medium at

37°C; B: YPD medium at 45°C; C: YPD medium supplemented with 40 μg/ml hygromycine B; D: YPD medium supplemented with 0.4% sodium deoxycholate; and E: YPD medium supplemented with 7 μg/ml Calcofluor white);

Fig. 5 shows results of HPLC analysis for size distribution and structure of sugar chains attached to glucose oxidase (GOD) expressed in an *H. polymorpha Hpoch2Δ* mutant strain, wherein the left panel (A, B and C) and right panel (D, E and F) represent results for sugar chains attached to GOD expressed respectively in an *H. polymorpha* wild type and the *Hpoch2Δ* mutant (A and D: sugar chain profiles attached to GOD; B and E: sugar chain profiles after treatment with α-1,2-mannosidase; and C and F: sugar chain profiles after subsequent treatment with α-1,6-mannosidase), and retention times of standard oligosaccharides of known size and structure are indicated by arrows (M5: $Man_5GlcNAc_2$-PA; M6: $Man_6GlcNAc_2$-PA; M8: $Man_8GlcNAc_2$-PA; and M11: $Man_{11}GlcNAc_2$-PA);

Fig. 6 shows results of tests for functional compensation of a *S. cerevisiae och1Δ* mutant by introduction of *HpOCH2* gene (1: *S. cerevisiae* wild type transformed with a control vector YEp352GAPII; 2: *S. cerevisiae och1Δ* mutant (*Scoch1Δ*) transformed with the control vector; and 3, 4 and 5: *Soch1Δ* mutant transformed respectively with an *HpOCH1* gene expression vector YEp352GAPII-HpOCH1 (3), an *HpOCH2* gene expression vector YEp352GAPII-HpOCH2 (4), and a *ScOCH1* gene expression vector YEp352GAPII-ScOCH1(5)), wherein yeast cultures (OD_{600}=1) which had arrived at an exponential phase

were 10-fold serially diluted, and 3 μl of each dilution was spotted on a YPD plate and cultured at 25℃ and 30℃ for three days (the A of Fig. 6); and gylcosylation of invertase expressed in each transformant was detected by activity staining (the B of Fig. 6);

Fig. 7 shows results of tests for measuring α-1,6-mannosyltransferase activity of *HpOch2* protein, wherein a *S. cerevisiae och1△mnn1△mnn4△* mutant strain was transformed with a control vector YEp352GAPII (A), an *HpOCH2* gene expression vector YEp352GAPII-HpOCH2 (B) or a *ScOCH1* gene expression vector YEp352GAPII-ScOCH1 (C), and membrane fractions obtained from the mutant strain were reacted with $Man_8GlcNAc_2$-PA at 30℃ for two hours and then analyzed by HPLC;

Fig. 8 shows results of HPLC analysis for size distribution and structure of sugar chains attached to GOD expressed in *H. polymorpha* strains
(A and D: sugar chain profiles of GOD expressed in a secretory form respectively in a *H. polymorpha* wild type and an *Hpoch2△* mutant; B and E: sugar chain profiles of GOD expressed respectively in a *H. polymorpha* wild type and an *Hpoch2△* mutant which are genetically engineered to express α-1,2-mannosidase derived from *Aspergillus saitoi*; and C and F: sugar chain profiles after treatment with α-1,2-mannosidase for sugar chains respectively from the recombinant wild type and mutant); and

Fig. 9 shows amino acid sequence identity and similarity between Och1 protein homologues of *S. cerevisiae*

and *H. polymorpha*.

Best Mode for Carrying Out the Invention

In eukaryotes, protein N-glycosylation starts in the endoplasmic reticulum (ER), where an N-linked oligosaccharide moiety of ($Glc_3Man_9GlcNAc_2$) is transferred to an appropriate asparagine residue of a nascent protein. From $Glc_3Man_9GlcNAc_2$, three glucose residues and one specific α-1,2-linked mannose residue are removed by specific glucosidases and α-1,2-mannosidase in the ER, resulting in the core oligosaccharide structure, $Man_8GlcNAc_2$. The protein with this core sugar structure is transported to the Golgi apparatus where the sugar moiety undergoes various modifications by various specific enzymes. In yeasts, the modification of the sugar chain in the Golgi apparatus involves a series of additions of mannose residues by different mannosyltransferases. The structure of the outer chain in N-glycosylation is specific to yeast species, typically with more than 50 mannose residues in *S. cerevisiae*.

The present inventors cloned an *HpOCH2* gene in a *H. polymorpha* strain DL-1 based on the complete genome information for *H. polymorpha*, and identified that the above gene has the activity of α-1,6-mannosyltransferase responsible for the outer chain initiation. The identified gene has a nucleotide sequence designated as SEQ ID NO. 1, and its corresponding amino acid sequence is designated as

SEQ ID NO. 2.

In one aspect, the present invention provides a nucleic acid molecule encoding for a protein designated as SEQ ID NO. 2. In addition, the present invention provides a nucleic acid molecule coding for a protein a protein with α-1,6-mannosyltransferase activity having an amino acid at least 75% homologous to the amino acid sequence of SEQ ID NO. 2. Preferably, the present invention provides a nucleic acid sequence designated as SEQ ID NO. 1, an analogue thereof or a fragment thereof.

The term "homologous", as used for a α-1,6-mannosyltransferase gene derived from *H. polymorpha* in the present invention, is intended to indicate the degree of similarity to the nucleotide sequence of a wild type, and includes a DNA sequence having an identity of preferably 75% or higher, more preferably 85% or higher, and most preferably 90% or higher, with a DNA sequence coding for α-1,6-mannosyltransferase. This homology comparison may be performed manually or by using a commercially available comparison program. A commercially available computer program may express homology between two or more sequences as a percentage, and a homology (%) may be calculated for adjacent sequences.

The present inventors registered the above gene at GenBank under accession number AY502025, and deposited a recombinant vector containing the gene, pBS-HpOCH2/Escherichia coli DH5α, at KCTC (Korean Collection for

Type Cultures; KRIBB, 52, Oun-dong, Yusong-ku, Taejon, Korea) on January 15, 2004, under accession number KCTC 10583BP.

Thus, in another aspect, the present invention provides a recombinant vector comprising a nucleic acid coding for a protein designated as SEQ ID NO. 2 or a protein with α-1,6-mannosyltransferase activity having an amino acid sequence at least 90% homologous to the amino acid sequence of SEQ ID NO. 2. The recombinant vector preferably comprises a DNA gene designated as SEQ ID NO. 1. In a further aspect, the present invention provides a host cell transformed with the recombinant vector, and preferably, provides a transformed host cell deposited under accession number KCTC 10583BP.

To produce a glycoprotien having a mammalian-type sugar chain in a yeast, a mutant strain yeast should be established, which lacks an enzyme family involved in yeast outer chain biosynthesis. Such a mutant strain may be attained by genetic mutation such as use of a reagent, ultraviolet illumination or spontaneous mutation, or by artificially disrupting a target gene. In the present invention, a gene (Och2) encoding α-1,6-mannosyltransferase playing a critical role in the outer chain initiation is disrupted by genetic engineering methods, that is, a combination of polymerase chain reaction and *in vivo* DNA recombination.

The present inventors established a *Hansenula polymorpha Hpoch2Δ* mutant strain (*Hansenula polymorpha* DL-1

och2△) in which a α-1,6-mannosyltransferase gene identified as described above is deficient, and found that yeast-specific consecutive addition of α-1,6-mannose residues is prevented in the mutant strain, so that hyperglycosylation is remarkably reduced. The mutant strain was deposited at KCTC (Korean Collection for Type Cultures; KRIBB, 52, Oun-dong, Yusong-ku, Taejon, Korea) on January 15, 2004, under accession number KCTC 10584BP.

Thus, in yet another aspect, the present invention provides a *Hansenula polymorpha Hpoch2△* mutant strain (*Hansenula polymorpha* DL-1 och2△) deposited under accession number KCTC 10584BP.

The majority of N-glycans on glycoproteins transported from the ER have a $Man_8GlcNAc_2$ sugar chain structure. After a protein is transported to the Golgi apparatus from the ER, additional mannose residues are added to the protein by different mannosyltransferases, resulting in a glycoprotien having numerous mannose sugar chains. The hyperglycosylation is undesirable in recombinant glycoproteins. Such hyperglycosylation may be reduced by using the *H. polymorpha* mutant strain prepared in the present invention as a host cell for the expression of recombinant proteins. In addition, when the *H. polymorpha* mutant strain is transformed with an expression vector capable of expressing one or more proteins having an enzymatic activity involved in sugar chain modifications, the hyperglycosylation may be more effectively inhibited or be converted to a sugar chain with a different structure. Sugar

chain-modifying enzymes involved in such hyperglycosylation reduction include α-1,2-mannosidase, mannosidase IA, mannosidase IB, mannosidase IC, mannosidase II, N-acetyl glucosaminyltransferase I, N-acetyl glucosaminyltransferase II, galactosyltransferase, sialyltransferase, and fucosyltransferase. However, the present invention is not limited to the above examples, and various genes capable of leading to a reduction and modification in hyperglycosylation of a recombinant glycoprotein may be also used. In an embodiment of the present invention, when α-1,2-mannosidase was expressed in the *H. polymorpha* Hpoch2△ mutant strain, a recombinant glycoprotein on which a yeast-type N-glycan was prevented from being formed and modified to a human-type N-glycan was produced. α-1,2-mannosidase removes a α-1,2-linked mannose residue at a non-reduced terminal of $Man_8GlcNAc_2$ and converts a core sugar chain on this glycoprotein to $Man_5GlcNAc_2$. The $Man_5GlcNAc_2$ structure is an inferior substrate for Golgi-residing mannosyltransferases, leading to a glycoprotein having reduced mannose content. The sugar chain-modifying enzyme gene contained in the expression vector used in the transformation may be the whole gene sequence encoding such an enzyme or a fragment sequence encoding a functional region of the enzyme. The expression vector includes an integrative or inductive promoter and a 3' termination sequence, and may be an integrative or replicative vector.

Thus, in still another aspect, the present invention provides a *H. polymorpha* mutant strain further comprising an

expression vector expressing a sugar chain-modifying enzyme. Preferably, the sugar chain-modifying enzyme is selected from the group consisting of α-1,2-mannosidase, mannosidase IA, mannosidase IB, mannosidase IC, mannosidase II, N-acetyl glucosaminyltransferase I, N-acetyl glucosaminyltransferase II, galactosyltransferase, sialyltransferase, and fucosyltransferase.

In still another aspect, the present invention provides a process for producing a recombinant glycoprotein with reduced glycosylation using an *H. polymorpha* mutant strain deposited under accession number KCTC 10584BP.

According to the present process as described above, a recombinant glycoprotein may be produced in a manner such that formation of a yeast-type N-glycan is prevented and the yeast-type N-glycan is modified to a human-type N-glycan.

The term "glycoprotein", as used herein, refers to a protein that is glycosylated on one or more asparagines, or one or more serine or threonine residues, or is glycosylated on asparagine and serine or threonine residues when expressed in a methylotropic yeast, particularly *Hansenula polymorpha*. The term "reduced glycosylation", as used herein, means that, when a glycoprotein is expressed in a methylotropic yeast strain, it has a reduced size of a carbohydrate moiety, particularly lower mannose residues, in comparison with the case of being expressed in a wild-type methylotropic yeast.

In the above process, a glycoprotein expression vector introduced into the *Hansenula polymorpha Hpcho2Δ* mutant strain preferably expresses a sugar chain-modifying enzyme

such as α-1,2-mannosidase along with glycoprotein.

A produced glycoprotein may be purified by a method commonly used in the art, and a purification protocol may be determined according to the properties of a specific protein to be purified. This determination is considered an ordinary skill to those skilled in the art. For example, a target protein may be purified by a typical isolation technique, such as precipitation, immunoadsorption, fractionization or various chromatographic methods.

Glycoproteins capable of being produced according to the present invention are exemplified by cytokines (e.g., interferon-α, interferon-β, interferon-γ, G-CSF, etc.), clotting factors (e.g., VIII factor, IX factor, human protein C), growth hormone releasing factor, *Penicillium minioluteum* dextranase, *Bacillus amyloliquefaciens* α-amylase, *Saccharomyces cerevisiae* aspartic protease, *Saccharomyces cerevisiae* invertase, *Typanosoma cruzi trans*-sialidase, HIV envelope protein, influenza virus A haemagglutinin, influenza neuraminidase, bovine enterokinase activator, bovine herpes virus type-1 glycoprotein D, human angiostatin, human B7-1, B7-2 and B-7 receptor CTLA-4, human tissue factor, growth factors (e.g., platelet-derived growth factor, endothelial growth factor), human α_1-antitrypsin, human antithrombin III, human erythropoietin, tissue plasminogen activators, plasminogen activator inhibitors, urokinase, α-galactosidase, plasminogen, thrombin, and immunoglobulins.

In still another aspect, the present invention provides a glycoprotein produced by the above process.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Identification of *Hansenula polymorpha HpOCH2* gene and analysis of the amino acid sequence of the gene

From the recently completed sequence of *Hansenula polymorpha* RB11 genome (Ramezani-Rad et al., FEMS Yeast Res., 4, 207-215 (2003)), whole sequences of ORFs (open reading frames) having a high similarity with the *OCH1* gene family involved in outer chain biosynthesis of *Saccharomyces cerevisiae* were obtained. Fig. 9 shows amino acid sequence identity and similarity between Och1 protein homologues of *H. polymorpha* and *S. cerevisiae*, wherein the amino acid sequence identity and similarity between ORF168 and ScOch1p are represented by shaded bold numerals.

In the present invention, for functional analysis of the ORF168 gene having a 40% amino acid identity and a 54% amino acid similarity with *S. cerevisiae OCH1* gene (*ScOCH1*) (Jungman and Munro, Embo J. 17, 423 (1998)) that plays a critical role in α-1,6-mannose addition at the early stage of outer chain biosynthesis of *S. cerevisiae*, polymerase chain reaction (PCR) was carried out using DNA extracted from *Hansenula polymorpha* DL-1 (Levine and Cooney, Appl. Microbiol., 26, 982-990,(1973)) as a template and a pair of primers(168Not-N and 168Not-C; Table 1). As a result, a

1.35-kb DNA fragment containing the ORF168 was obtained, and was then subjected to amino acid sequencing.

A conventionally identified *H. polymorpha* gene described in Korean Pat. Application No. 2002-37717 applied by the present inventors has an amino acid sequence having a 22% identity and a 36% similarity with *S. cerevisiae OCH1* gene and is thus designated as *HpOCH1*. In this regard, the ORF168 identified in this invention was designated as *HpOCH2*, and its nucleotide sequence was registered at GenBank under accession number AY502025. *HpOCH2* was 1287 bp long and expected to code for a protein consisting of 428 amino acids. HpOch2 protein had a potential transmembrane spanning region at a region from 29 to 51 positions, and was thus considered as a type II membrane protein to which most glycosyltransferases belong (Fig. 1). Also, HpOch2 protein was observed to have a DXD element known as an active site of glycosyltransferases (Lussier et al., J. Cell. Biol., 131, 913-927, (1995)), and was thus expected to have glycosyltransferase activity (Fig. 1). The amino acid sequence of HpOch2 protein was found to have a relatively high similarity with (around 40% identity), in addition to a *S. cerevisiae OCH1* gene product, *OCH1* gene products of other yeasts, that is, *Candida abicans*: Thomas et al., unpublished results, GenBank accession number AY064420), *Pichia pastoris*: Japanese Pat. 07145005), *Schizosaccharomyces pombe*: Yoko-o et al., FEBS Lett., 489, 75-80, (2001)) (Fig. 2).

EXAMPLE 2: Establishment of *H. polymorpha HpOCH2* gene-

deficient strain and analysis of characteristics of the strain

To establish a *H. polymorpha HpOCH2* gene-deleted mutant strain, gene disruption was performed by a combination of fusion PCR and *in vivo* DNA recombination (Oldenburg *et al.*, Nucleic Acid Res., 25, 451, (1997)). Fusion PCR was carried out using primers (primers used for PCR for cloning and disruption of *HpOCH2* gene) listed in Table 1, below. By primary PCR, 5′ and 3′ regions of *URA3* gene and *HpOCH2* gene were obtained. By secondary fusion PCR, the 5′ region of *HpOCH2* gene was linked to the 5′ region of *URA3* gene, and the 3′ region of *URA3* gene was liked to the 3′ region of *HpOCH2* gene. Then, the two DNA fragments were introduced into a yeast cell, and transformants having an *HpOCH2* gene disrupted by *in vivo* DNA recombination were selected (Fig. 3). Primarily, using an *URA3* selection marker, transformants grown in a minimum medium lacking uracil were selected. Then, amplified DNA fragments produced by PCR were examined to determine whether they differ from those of a wild-type strain, thereby selecting a *H. polymorpha* mutant strain having a different amplification pattern, *Hpoch2Δ (leu2 och1::URA3)*. The obtained *Hpoch2Δ* strain was evaluated for growth properties. The *Hpoch2Δ* strain was found to have temperature sensitivity at 45°C like a *Hpoch1Δ* strain (KCTC 10264BP), but, unlike the *Hpoch1Δ* strain, had a similar growth rate to the wild type at 37°C. Also, growth was greatly inhibited in the presence of hygromycin B, but little

sensitivity to sodium deoxycholate was observed (Fig. 4). Since these growth properties are common in mutant strains having a defect in outer chain synthesis, the *H. polymorpha Hpoch2*△ strain was believed to have a defect in the outer chain glycosylation process.

TABLE 1

Primer	Sequences	SEQ ID. No.
168Not-N	5'-AAGGAAAAAAGCGGCCGCCGGTGAAGAATGGTGTAT-3'	3
168Not-C	5'-TTTTCCTTTTGCGGCCGCCGTTCTGTGCCTGCTCATGAT-3'	4
UNfor	5'-GGATCCCCGGGTACCGAGCT-3'[a]	5
UNrew	5'-CACCGGTAGCTAATGATCCC-3'	6
UCfor	5'-CGAACATCCAAGTGGGCCGA-3'	7
UCrew	5'-CTGGCGAAAGGGGGATGTGC-3'[b]	8
168Nfor	5'-GGCGGATATGGGGCTTCGCC-3'	9
168Nrew	5'-AGCTCGGTACCCGGGGATCCCGTTCCAGGGCTCCACGTCC-3'[c]	10
168Cfor	5'-GCACATCCCCCTTTCGCCAGCCGATCACGAGCTTCAGTCC-3'[d]	11
168Crew	5'-CGTCGTCCGGGCCCAGTTCG-3'	12

EXAMPLE 3: Analysis of size distribution and structure of sugar chains on a glycoprotein synthesized in the *H. polymorpha Hpoch2*△ mutant

To analyze the size distribution and structure of sugar chains on glycoprotein synthesized in the *H. polymorpha Hpoch2*△ mutant prepared in Example 2, a glycoprotein derived from *Aspergillus niger*, glucose oxidase (GOD), was expressed in a secreted form in a *H. polymorpha* wild type and the *Hpoch2*△ mutant. The glycoprotein, GOD,

has eight putative sites for *N*-linked glycosylation (Frederick et al., J. Biol.Chem., 265, 3793 (1990)). The *H. polymorpha* wild type and *Hpoch2*△ mutant were individually transformed with an expression vector pDLMOX-GOD(H) expressing GOD with a six-histidine tag (Kim et al., Glycobiology (2004)), and were grown in YPM medium (1% yeast extract, 2% peptone, 2% methanol) to express GOD. GOD secreted to the culture medium was passed through a nickel column to selectively isolate only GOD tagged with six histidines at the C-terminal region. The isolated recombinant his-tagged GOD was treated with PNGase F to detach attached sugar chains from the GOD. Then, the released sugar chains were labelled with 2-aminopyridine (2-PA) and subjected to HPLC analysis. As shown in the A and D of Fig. 5, sugar chains of wild type-derived recombinant GOD were found to have various size distributions ranging from 8 to 12 mannose residues. In contrast, sugar chains attached to recombinant GOD expressed in the *Hpoch2*△ mutant were found to mostly have core sugar chains with 8 mannose residues. These results indicate that sugar addition after the eighth mannose residue is greatly inhibited in the *Hpoch2*△ mutant. Separately, the sugar chains released from recombinant GOD were treated sequentially by α-1,2-mannosidase and α-1,6-mannosidase to investigate changes in a sugar chain profile. Sugar chains synthesized by the wild type were converted to sugar chains corresponding to five or six mannose by α-1,2-mannosidase, and all of them were then converted to sugar chains corresponding to five mannose by

α-1,6-mannosidase. In contrast all sugar chains of the *Hpoch2△* mutant were converted to the sugar chains corresponding to five mannose by only α-1,2-mannosidase (Fig. 5). These results reveal that initiation of outer chain elongation via α-1,6-mannose linkage never occurs in the *Hpoch2△* mutant, thereby indicating that the *HpOCH2* gene product is directly or indirectly involved in the activity of α-1,6-mannosidase.

EXAMPLE 4: Functional analysis of *H. polymorpha* HpOCH2 protein

To determine whether the *H. polymorpha HpOCH2* gene product is a functional homologue to *S. cerevisiae* Och1 protein adding α-1,6 mannose to a core sugar chain in the initiation process of outer chain synthesis, an expression vector carrying an *HpOCH2* gene, YEp352GAPII-HpOCH2, was introduced into a mutant strain having a disruption in *S. cerevisiae OCH1* gene, *Scoch1△*, and the *Scoch1△* mutant was evaluated for ability to overcome thermosensitivity (the A of Fig. 6). *HpOCH2*, *HpOCH1* and *ScOCH1* genes were individually inserted between glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and terminator introduced into a *S. cerevisiae* expression vector YEp352 (Hill et al., Yeast, 2, 163-167, (1986)), thus generating expression vectors, YEp352GAPII-HpOCH2, YEp352GAPII-HpOCH1 and YEp352GAPII-ScOCH1, respectively. The *S. cerevisiae Scoch1△* mutant, when transformed with the *HpOCH2* gene expression vector

(YEp352GAPII-HpOCH2), recovered its ability to grow at high temperature. By contrast, when transformed with the expression vector (YEp352GAPII-HpOCH1) carrying *HpOCH1* gene having a nucleotide sequence similarity to *HpOCH2* gene, the *Scoch1*△ mutant did not overcome thermosensitivity (the A of Fig. 6). In addition, the *Scoch1*△ mutant was evaluated for another feature of having a defect in hyperglycosylation. When the *HpOCH2* expression vector was introduced into the *Scoch1*△ mutant, as shown in the B of Fig. 6, the glycosylation of a glycoprotein, invertase, recovered to a level identical to that when the *S. cerevisiae OCH1* expression vector (YEp352GAPII-ScOCH1) was introduced thereinto (Fig. 6B, lines 4 and 5). By contrast, when the *HpOCH1* expression vector was introduced into the *Scoch1*△ mutant, the defect in glycosylation of invertase was unchanged. The results, that the *S. cerevisiae och1*△ mutant overcomes thermosensitivity and recovers hyperglycosylation by the expression of *H. polymorpha HpOCH2* gene, demonstrate that the *HpOCH2* gene product is a functional homologue to *S. cerevisiae* Och1 protein playing a critical role in the first step of outer chain biosynthesis.

An *in vitro* assay was performed to determine whether the *H. polymorpha* HpOch2 protein practically has α-1,6-mannosyltransferase activity to add α-1,6-mannose to a core sugar chain like the *S. cerevisiae* Och1 protein. A mutant strain (*och1*△*mnn1*△*mnn4*△) disrupted in three genes, *OCH1*, *MNN1* and *MNN4*, has a complete loss of outer chain synthesis (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)).

The *och1△mnn1△mnn4△* mutant was transformed with the *H. polymorpha HpOCH2* gene expression vector, and a membrane fraction was prepared. The membrane fraction was used as an enzyme source for measuring α-1,6-mannosyltransferase and reacted with a substrate having a core sugar chain structure, $Man_8GlcNAc_2$-PA. The resulting reaction solution was analyzed by HPLC. When the *och1△mnn1△mnn4△* mutant was transformed with the Yep352GAPII vector not containing *HpOCH2* gene, the concentration of the substrate $Man_8GlcNAc_2$-PA was not changed in a membrane fraction. By contrast, when the *och1△mnn1△mnn4△* mutant was transformed respectively with the *H. polymorpha HpOCH2* gene expression vector and the *S. cerevisiae OCH1* gene expression vector, a peak corresponding to $Man_9GlcNAc_2$-PA (a structure formed by the addition of a single mannose to $Man_8GlcNAc_2$-PA) was observed in membrane fractions (Fig. 7). These results indicate that the *H. polymorpha* HpOch2 protein, like the *S. cerevisiae* Och1 protein, has the activity of α-1,6-mannosyltransferase involved in the initiation of outer chain elongation.

EXAMPLE 5: Glycotechnology using the *H. polymorpha Hpoch2△* mutant

An *H. polymorpha* strain capable of producing a recombinant glycoprotein having a human mannose-type *N*-linked glycan was established as follows. As described in a previous study (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)) carried out with the traditional yeast *S.*

cerevisiae, the *H. polymorpha Hpoch2*△ mutant was transfected with a α-1,2-mannosidase expression vector for application to *H. polymorpha*, pDUMOX-MsdS(HA-HDEL), in order to express *Aspergillus saitoi* α-1,2-mannosidase in the ER of *H. polymorpha*, thereby developing a glycoengineered recombinant strain *Hpoch2*△-MsdSp. To construct the α-1,2-mannosidase expression vector, pDUMOX-MsdS(HA-HDEL), PCR was carried out using a plasmid containing *Aspergillus saitoi* α-1,2-mannosidase, pGAMH1 (Chiba et al., J. Biol. Chem., 273, 26298-26304, (1998)), as a template and a forward primer (5'-GGGGAATTCAAAAAAATGGTGGTCTTCAGCAAA-3': SEQ ID. NO. 13) containing an EcoRI site, and a reverse primer (5'-GGGCCATGGTCACAATTCATCATGCGCATAGTCAGGAACATCGTATGGGTATGTACTACTC ACCCGCAC-3': SEQ ID. NO. 14) containing an HA sequence for determining protein expression levels, an HDEL (His-Asp-Glu-Leu) sequence as an endoplasmic reticulum retention/retrieval tag and a NcoI site. As a result, the *A. saitoi* α-1,2-mannosidase was amplified, thus yielding a 1.5-kb fragment. The 1.5-kb fragment was digested with EcoRI and NcoI and replaced a GOD gene of a GOD expression vector pDLMOX-GOD(H) (Kim et al.. Glycobiology, 14, 243-251, (2004)). Then, in the resulting GOD expression vector, an *H. polymorpha LEU2* selection marker was replaced by an *H. polymorpha URA3* selection marker, thus finally yielding the α-1,2-mannosidase expression vector pDUMOX-MsdS(HA-HDEL). The expression of *A. saitoi* α-1,2-mannosidase in *H. polymorpha* was detected by Western blotting using an anti-HA antibody (Sigma).

To determine whether the glycoengineered *H. polymorpha* strain *Hpoch2*△-MsdSp synthesizes a human mannose-type *N*-glycan, the structure of sugar chains attached to GOD expressed in a secreted form was analyzed. In a recombinant wild-type strain, *HpOCH2*-MsdSp, transformed with a heterogeneous α-1,2-mannosidase, sugar chains with 8 or higher mannose residues were sharply reduced, while sugar chains with 5 or 6 mannose residues were increased (the A and B of Fig. 8). In the recombinant *Hpoch2*△-MsdSp strain prepared by transforming the *Hpoch2*△ strain with a heterogeneous α-1,2-mannosidase, sugar chains with more than 7 mannose residues were reduced in comparison with the recombinant wild-type strain *HpOCH2*-MsdSp (the B and E of Fig. 8). When sugar chains isolated from the recombinant wild-type strain and the *Hpoch2*△ mutant strain were treated with α-1,2-mannosidase, the sugar chains of the *HpOCH2*-MsdSp strain were converted to sugar chains with 5 and 6 mannose residues, whereas sugar chains of the *Hpoch2*△-MsdSp strain were converted to sugar chains with 5 mannose residues by α-1,2-mannosidase (the C and F of Fig. 8). These results reveal that, when the heterogeneous α-1,2-mannosidase is introduced into *H. polymorpha*, a wild-type strain still forms yeast-specific α-1,6-mannose linkage by *H. polymorpha* HpOch2 protein, thereby indicating that, to synthesize human mannose-type *N*-glycans, *H. polymorpha HpOCH2* gene should be essentially disrupted. Therefore, the *Hpoch2*△ mutant strain having a deficient *HpOCH2* gene, developed in the present invention, is useful as a host for the production of

therapeutic recombinant glycoproteins having human compatible sugar chains. In addition, when various sugar chain-modifying enzymes are expressed in the *Hpoch2*△ mutant strain, they come to have various sugar moieties that are not immunogenic in the human body. Thus, the *Hpoch2*△ mutant strain is very useful in glycotechnology for the development of a host producing a novel glycoprotein having increased physiological activity as well as having non-immunogenic sugar moieties.

Industrial Applicability

Since *H. polymorpha* has been approved worldwide as a host system for mass production of recombinant hepatitis vaccines, recombinant proteins to be expressed in *H. polymorpha* have a high potential to be developed as therapeutics. As described in the above Examples, in the *Hpoch2*△ mutant strain having a deficiency in *H. polymorpha HpOCH2* gene, developed in the present invention, the initiation of outer chain elongation is prevented, resulting in the prevention of yeast-specific consecutive α-1,6-mannose addition. Thus, the *H. polymorpha* mutant strain can be used as a host to produce a target glycoprotein in the form of having a sugar chain structure closer to that of human glycoproteins via a secretory pathway. Also, as described above, the *Hpoch2*△ mutant strain becomes a basis in glycoengineering for the development of various *H. polymorpha* strains which may be used as hosts for the mass

production of recombinant glycoproteins of therapeutic value. Therefore, the present *Hpoch2*Δ mutant strain is very useful in related industrial fields.

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